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Development of Bacterial Display Peptides for use in Biosensing Applications

by Dimitra N. Stratis-Cullum, Joshua M. Kogot, Michael S. Sellers, Margaret M. Hurley, Deborah A. Sarkes Joseph M. Pennington, Irene Val-Addo, Bryn L. Adams, Candice R. Warner, James P. Carney, Rebecca L. Brown, and Paul M. Pellegrino

ARL-RP-0404 **September 2012**

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14. ABSTRACT

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15. SUBJECT TERMS

peptide, synthetic reagent, affinity reagent, bacterial display, multi-scale modeling, docking, protective

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Development of bacterial display peptides for use in biosensing applications

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ABSTRACT

Recent advances in synthetic library engineering continue to show promise for the rapid production of reagent technology in response to biological threats. A synthetic library of peptide mutants built off a bacterial host offers a convenient means to link the peptide sequence, (i.e., identity of individual library members) with the desired molecular recognition traits, but also allows for a relatively simple protocol, amenable to automation. An improved understanding of the mechanisms of recognition and control of synthetic reagent isolation and evolution remain critical to success. In this paper, we describe our approach to development of peptide affinity reagents based on peptide bacterial display technology with improved control of binding interactions for stringent evolution of reagent candidates, and tailored performance capabilities. There are four key elements to the peptide affinity reagent program including: (1) the diverse bacterial library technology, (2) advanced reagent screening amenable to laboratory automation and control, (3) iterative characterization and feedback on both affinity and specificity of the molecular interactions, and (3) integrated multi-scale computational prescreening of candidate peptide ligands including *in silico* prediction of improved binding performance. Specific results on peptides binders to Protective Antigen (PA) protein of Bacillus anthracis and Staphylococcal Enterotoxin B (SEB) will be presented. Recent highlights of on cell vs. off-cell affinity behavior and correlation of the results with advanced docking simulations on the protein-peptide system(s) are included. The potential of this technology and approach to enable rapid development of a new affinity reagent with unprecedented speed (less than one week) would allow for rapid response to new and constantly emerging threats.

Keywords: peptide, synthetic reagent, affinity reagent, bacterial display, multi-scale modeling, docking, protective antigen, SEB, biosensing

1. INTRODUCTION

The challenge in hazard detection (whether chemical or biological in nature) is detection of trace concentrations of materials in a complex background plagued with interferences. The Army and broader sensing community are in need of technologies that can offer robust, rapid, and reliable detection. The "heart" of any biosensor technology is the bioreceptor element (i.e. affinity reagent), as it is responsible for the specific recognition of the target threat of interest. The current state-of-the-art in element technology (i.e., antibody bioreceptors) is fraught with difficulties including: poor mass production capabilities, reagent stability, and large overall production costs. Moreover, the rate at which the DoD requires reagents for new and emerging threats far exceeds the rate at which they can currently be produced. Antibodies usually require several weeks-to-months to isolate from living hosts whereas recent advances have demonstrated that synthetic routes can be employed using various synthetic recognition element technologies (e.g., bacterial display and phage display) to produce bioreceptors in as little as a few days to a couple weeks (see Figure 1).[1-3]

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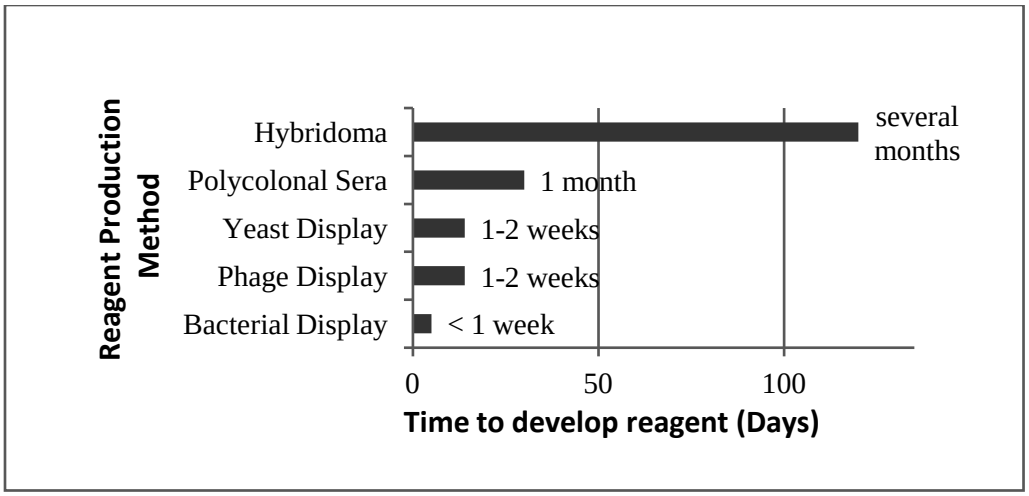

Figure 1. Comparison of time needed to develop new reagents using antibody and synthetic protein regent technologies.

The mechanisms for specific binding from a synthetic recognition element (SRE) receptor are similar to antibody-antigen interactions but unlike antibodies, peptide reagents can be rapidly developed through bio-engineered library screening toolkits and mass-produced through standard synthesis techniques. Another potential advantage of peptide-based sensing includes a greater stability that would allow for wide-spread field use, a shortcoming of current antibody technology. Several variations of peptide development technologies including yeast, phage, and bacterial display are currently being used to isolate SREs. However, with bacterial peptide display technology, the extremely fast replication rate of bacteria is exploited and biological components (e.g., modified proteins) on an *E. coli* cell surface are harnessed to produce the peptide library of binders. It is with this speed of bacterial growth rates, combined with improved computational techniques to characterize and tailor peptide affinity and specificity, that bacterial peptide display technology has emerged as a means to rapidly isolate high affinity protein binders (within 1 week). This is critical for combating newly emerging biological weapons of mass destruction (WMD) that have no readily available or specific antibody bioreceptor.

In Figure 2, we show there are four key research areas that we bring together for the development of peptide SREs for affinity reagent discovery including: (1) the diverse bacterial library technology, (2) advanced reagent screening amenable to laboratory automation and control, (3) iterative characterization and feedback on both affinity and specificity of the molecular interactions, and (3) integrated multi-scale computational prescreening of candidate peptide ligands including *in silico* prediction of improved binding performance.

To engineer a "library" of peptide binding elements, a small section of protein from the surface of a biological system (e.g., bacterium, virus, etc.) is manipulated to present a randomized sequence of amino acids (the building blocks of proteins). Previous work by the Daugherty lab, details the development and early screening results with the eCPX platform[4]). Briefly, this peptide library employs an *E. coli* bacterial display platform, generated from the extracellular loop of OmpX outer-membrane protein. The randomized portion of the library is a 15-mer, yielding a greater than 10^{10} member library. The bacterial display library utilized throughout these studies is an enhanced form, termed eCPX. The OmpX outer membrane protein has been engineered into a circularly permuted scaffold to allow both C-terminal and N-terminal display of proteins.

The large diversity of sequences (10^{10} members for bacterial display presented by this library) is then exposed to a target of interest and stringency controls, in an alternating fashion. This procedure is sometimes referred to as "bio-panning," "library screening," or "sorting" and is conducted to isolate a small subset of peptide sequences that demonstrate extremely high binding affinity and specificity. Sorting, or screening of peptide libraries for suitable reagent candidates is typically performed by a combination of several rounds of manual MACS for pre-enrichment and several rounds of FACS. Although effective, the approach is quite labor intensive, and most importantly the performance characteristics of the resulting reagents are highly operator dependent. Furthermore, the cost of an instrument equipped with fluorescence

activated cell (FACS) sorting and isolation is often prohibitively expensive (~$350-500K) for many laboratories. Consequently the ability to automate the process on an inexpensive platform is highly desired.

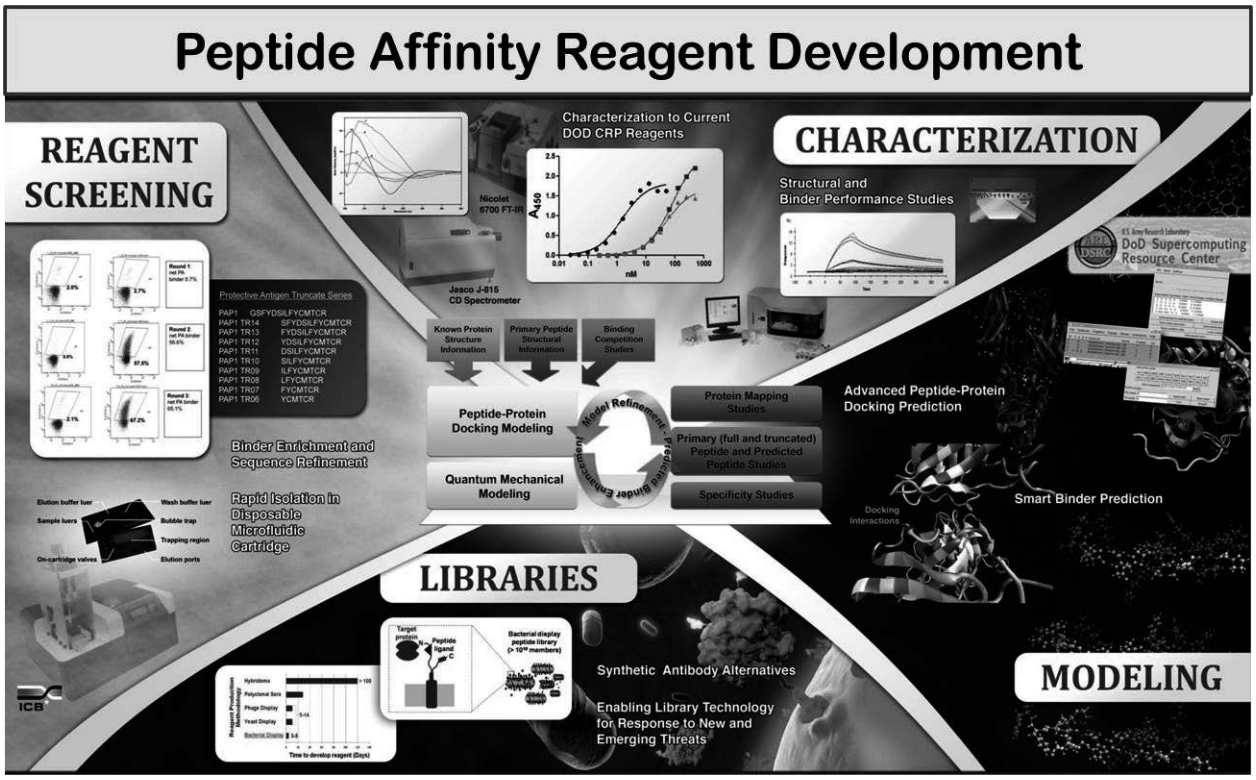

Figure 2. Schematic diagram showing the four main areas of research which enable rapid, high-performance synthetic recognition element development.

The key performance metrics to SRE isolation include the purity of separation (ability to perform a pure separation of the binding reagents from background), rare-cell recovery (ability to isolate extremely low cell count populations from the sea of 10^{10} binder library cells), and throughput (ability to sort several mL of library solution in minutes for high throughput isolation). We recently published on our work showing rare-cell recovery and reagent studies using the second generation Micro Magnetic Sorter (MMS), which consists of a semi-automated magnetic separation system consisting of a disposable microfluidic cartridge. SRE reagent screening with the MMS approach was found to cause an increase in reproducibility and control over the biopanning with consistent results through cross-laboratory study.[1]

Characterization of binder performance is a critical component of SRE development as it allows for isolation of high affinity peptides for the target of interest but more importantly specific reagents, which is directly linked to the degree to which the sensor, ultimately using this reagent will false alarm. On-cell characterization is typically performed using flow cytometry analysis. Off-cell characterization is significantly more challenging due to the small size of the peptides where chemical modification can significantly alter the function and performance and solubility of the synthetic peptide is sometimes problematic. Recently, we published a custom method[5] developed to address these challenges by incorporating a fusion tag which binds to polystyrene. The polystyrene fusion tag provides an overall better solubility profile and a means by which to incorporate the peptide as a reagent into a standard enzyme-linked immunosorbent assay format. Although more expensive and less standard, off-cell characterization can also be performed label-free through other custom methods, such as surface Plasmon resonance spectroscopy (SPR).

2. MATERIALS AND METHODS

2.1 MMS Disposable Microfluidic Card:

The Micro-Magnetic Sorter (MMS) is an automated magnetic separation system consisting of a disposable microfluidic cartridge and a companion instrument (Figure 3). The disposable cartridges are made of injection-molded polypropylene (Pinnacle Polymers PP 5135C). The 200 µm deep fluidic channels are defined by two injected parts, which are laser-welded (California Lasers, Simi Valley, CA) and a portion is heat staked with a hydrophobic membrane for bubble removal (Pall Co, Ann Arbor, MI). The trapping region was designed to accommodate up to 1 x 10^9 of 1 µm trapped magnetic beads and process up to 1 x 10^{11} bacterial cells. Female luer fittings on the top of the cartridge allow for a leak-proof interface between the cartridge and disposable syringes (Becton Dickinson, San José, CA). The luer fittings on the cartridge are designed to hold a reservoir array, for pneumatically driven applications as well as the injector inputs. There are a total of four luer ports required for two sample injectors (1 or 5 mL volume), one running/wash buffer injector (up to 10 mL) and one elution buffer injector (up to 3 mL volume). Strategically designed micro-channels allow for full automation of magnetic separation on the cartridge. To accomplish this, five pneumatically actuated pinch valves are located on the underside of the cartridge, which allow for the redirection of flow. These valve membranes require a force of ~15 lb/in^2 to seal and are robust enough to be actuated multiple times.

2.2 MMS Instrumentation

The instrument utilizes a cRIO controller with LabVIEW script (National Instruments, Austin, TX) outfitted with standard digital and analog in/out modules for control of the internal components. Flow rates within the cartridge are controlled by four stepper motors (Figure 7c) and controller boards (Haydon and Anaheim Automation, respectively), which physically push on the injectors (Figure 7d). These motors are fitted with micro-switches (Panasonic ECG, Secaucus, NJ) that allow for the automatic calculation of input volume. Valves on the cartridge are actuated using pneumatically controlled air cylinders (SMC Corp, Noblesville, IN) and a DC diaphragm pump (Thomas provided by Nor Cal Controls, San Jose, CA). There are seventy custom neodymium-iron boron magnets, which are position-controlled by another Haydon stepper motor. The magnets are distributed equally among top and bottom portions of a magnetic rack, which sandwich the disposable cartridge. A single motor, in conjunction with a spring, allows for both horizontal and vertical movement of magnets. This facilitates horizontal movement required for trapping and elution, and vertical movement capable of agitating the sample within the cartridge. Software control is provided using a LabVIEW interface. Push button applications have been created for bacterial library sorting. Advanced users can generate custom sorting routines, which allow full access to all the operation parameters with minimal training. Direct control of flow rates, wash stringencies, and positive/negative selection criteria enables the end user to specify the magnetic bead and protocol of choice, and optimize it for applications beyond bacterial library sorting (i.e. cell culture, flow cytometry, toxicology studies, etc.).

2.3 Sorting Procedures and Sample Preparation

Figure 4 shows a general schematic of the library sorting scheme. A bacterial display library (Cytomx Therapeutics; San Francisco, CA: eCPX library) which contains approximately 3 × 10^{10} members was screened for clones that display PA binding peptides The random library is first grown in 500 mL LB media containing 25 µg/mL chloramphenicol (LB-Cm^{25}) to an OD_{600nm} of approximately 0.6 (Eppendorf Biophotometer; Eppendorf, Hamburg, Germany). At this point in exponential growth phase the cells were induced by the addition of arabinose to a final concentration of 0.04% (w/v); the enhanced circularly permuted OmpX (eCPX) gene expressing the library peptides is under the control of an arabinose inducible promoter (Rice, 2008). The cells were shaken at 37 °C for an additional 45 mins, after which the OD_{600nm} was again measured and, using the assumption that an OD_{600nm} of 1.0 relates to a bacterial concentration of 1 x 10^9 cfu/mL, approximately 2 x 10^{11} cells were pelleted by centrifugation at 3000g for 20 mins.

2.4 PA-binder enrichment

The SA-binder depleted library was centrifuged at 3000 g for 20 mins, resuspended in 1mL phosphate buffered saline with 0.5% bovin serum albumin (PBSB) buffer containing 600 nM biotinylated protective antigen, PA, (List Biological

Laboratories, Inc; Campbell, CA), and incubated at 4 ºC for 45 mins. Cells were centrifuged as above and re-suspended in 1 mL PBSB buffer with 1 x 10^9 pre-washed magnetic beads. After 45 mins at 4 ºC with rotation, the cell-beads suspension was loaded into an MMS cartridge (or separated by manual MACS using the same methods as SA binder depletion). Bacterial cells bound to PA were trapped on cartridge, and then eluted into a collection vessel. A second round of sorting was performed following the same protocol as the first; however, the assay parameters were adjusted to account for the smaller starting population and to increase the selection pressure in the second round, therefore 1 x 10^8 cells in 50 µL of 300 nM PA and 1 x 10^8 magnetic beads were used. Cells were incubated static on ice for all labeling steps. Also, 1 µM biotin was added in the washing buffer to compete with any remaining streptavidin binders (peptides which bind to streptavidin typically have a much lower affinity than biotin). In the third round of MMS sorting, cells were labeled with 150 nM biotinylated PA, and then labeled with 1 x 10^6 magnetic beads in 50 µL of PBSB. After each round of magnetic separation, the bead-bound enriched library was added to LB-Cm^{25} media supplemented with 0.2% glucose to inhibit expression of the eCPX gene and therefore prevent growth bias. The cultures were then grown overnight at 37 ºC with shaking.

2.5 Flow Cytometry Analysis of Binder Enrichment

To quantify the library enrichment of potential PA binders, flow cytometry analysis (BD FACSAria; BD Biosciences, Franklin Lakes, NJ) was performed using biotinylated PA (EZ-Link Sulfo-NHS biotinylation kit; Thermo Scientific, Rockford, IL) labeled with alternating fluorescent secondary labels: streptavidin, R-phycoerythrin conjugate (SAPE; Invitrogen, Carlsbad, CA), and Neutravidin, R-phycoerythrin conjugate (NAPE; Invitrogen, Carlsbad, CA). Following each round of PA selection, the arabinose induced cell population was incubated with 100 nM biotin-PA solution for 45 mins. The sample was centrifuged at 3000 x g for 10 mins to remove unbound biotin-PA and was resuspended in a 25 µL solution of PBSB with secondary label concentration of 5 µg/mL and incubated for 45 mins at 4 ºC. The sample was centrifuged and resuspended in 1 mL ice-cold BD FACSFlow (BD Biosciences, Franlin Lakes, NJ) sheath immediately prior to flow cytometry. Cells labeled with SAPE exhibit increased red fluorescence and are easily distinguishable by flow cytometry.

3. RESULTS AND DISCUSSION

Previously, we demonstrated the effectiveness eCPX technology for bacterial library sorting and the capability of automating the selection using the micromagnetic sorter (MMS) platform[1]. Advantages to this approach include reproducible reagent isololation in a disposable cartridge format to avoid exposure to potentially harmful threat materials under investigation. For a typical 1 mL sample volume, MMS requires only 5 mins of user interaction, while manual selection requires more than 20 mins. With reagent sorting, there are three key parameters that are critical to evaluate with the first being *throughput*, i.e. how many cells can be sorted per second. The MMS platform achieves high throughput screening since it is capable of screening a bacterial library containing 3×10^{10} members in 15 mins. With regard to gross throughput per hour, MMS is able to process 5×10^{12} cells/hr (50 mL/hr at a cell concentration of 1 x 10^{11} cells/mL), which is four orders of magnitude higher than that achieved using state-of-art FACS instrumentation or a previously reported dielectrophoretic cell sorter.[1] In this work, we compare peptide reagent candidates produced by the MMS sorter platform for targeting PA from *Bacillus anthracis*, and enterotoxin B from Staphylococcus aureus (SEB) in terms of binding affinity and specificity with specific focus on streptavidin cross-reactivity. We also show initial results on multi-scale docking simulations using the ARL XPairIt program developed in-house.

The importance of both affinity and specificity characterization is illustrated in Figure 3. In Figure 3a and d, flow cytometry analysis shows the negative control population. In figures 3b and 3e, a positive response to PA is clearly evident for both peptide reagent candidates. In figures 3c and f, the cross-reactivity with streptavidin is compared, and SM545 was found to have significantly more cross-reactivity with the PA target. In all cases the binding population is indicated in red and the overall percentage of the population determined as binding to the target or interferent is given. It is clear that there is very little binding to the cell or library scaffold itself, designated as the negative control panels 3a) and 3d) for the DS-28 and SM545 binders, respectively. It is clear upon examination of panels 5b and 5e, that both DS-28 and SM545 exhibit marked binding to the protective antigen target, with the SM545 binder exhibiting superior performance (95.4 % binding). Negative sorting is performed during the selection process to remove potentially cross-reactivity with the bead chemistry used for reagent isolation. However, it is necessary to evaluate the degree of cross-reactivity for any isolated reagent to have practical relevance. Figure 5 c and f show the cross-reactivity to streptavidin

for the clones DS-28 and SM545, respectively. From these data it is clear that both clones exhibit cross-reactivity to streptavidin and in the case of DS-28, the binding population to streptavidin is even greater than that to the target. The binding to the target PA over the streptavidin is significantly greater, however, for the SM545 clone at 95.4% (PA) and 24% (Streptavidin).

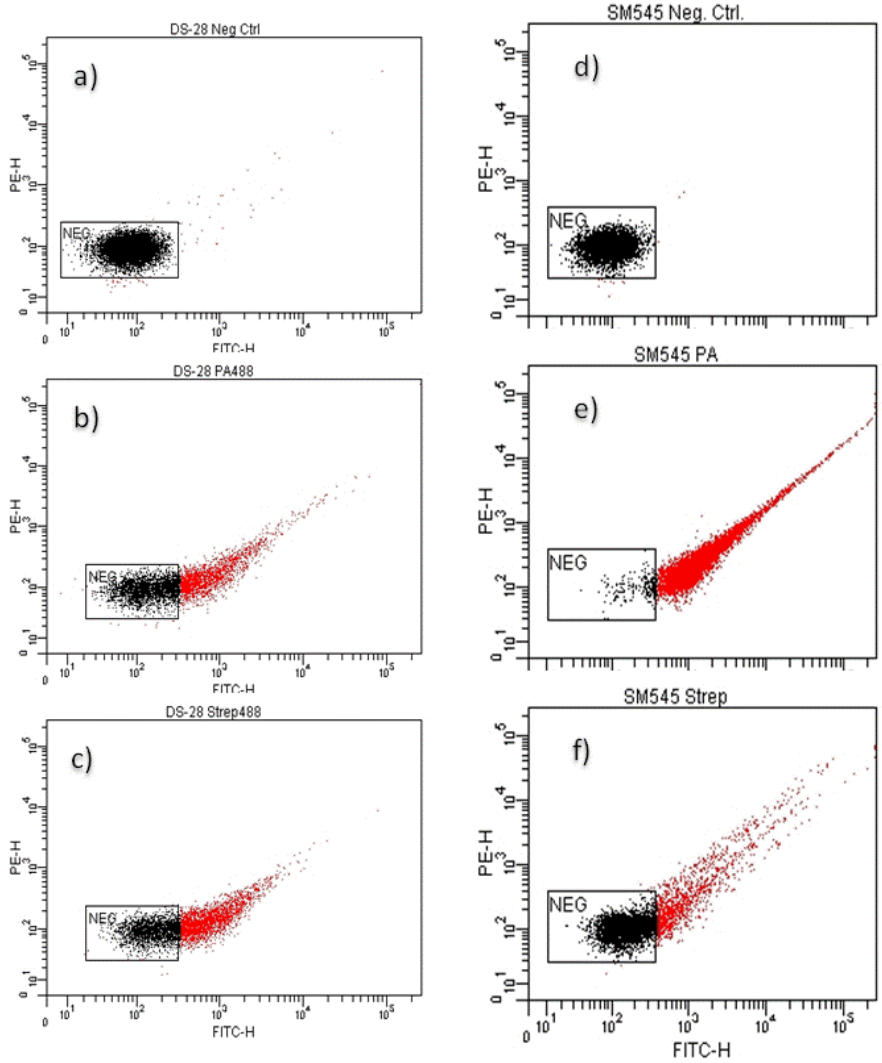

Figure 3: Flow cytometry analysis of two peptide reagent candidates designated DS-28 (a, b, c) and SM545, designated d, e, f. The binding to the cell scaffold alone is illustrated in a) for DS-28 and d) for SM545. The binding to the PA target is illustrated in b and c and c) and f) represents the fraction which binds to streptavidin. In all panels, the binding population is indicated in red.

Through a streptavidin pre-sort, we are able to cleanse the binder population of significant streptavidin binding and have adopted this streptavidin depletion coupled with *in silico* optimization of binding affinity subsequent to selection as a general strategy for development of both specific and high affinity peptide reagent candidates. An example of the improved specificity for PA is shown in Figure 4, upon comparison of the FACS characterization of clone DS23 produced from the pre-sorted library binding to PA (4a) and Streptavidin (4b). The percent of the population binding was calculated to be 68.8% and 1.6% for PA and Streptavidin, a marked improvement in specificity.

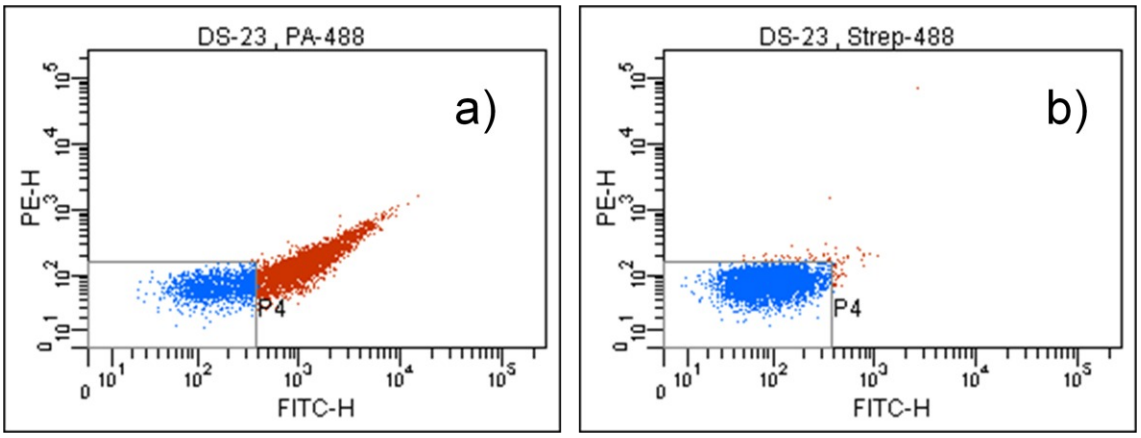

Figure 4: Flow cytometry analysis of the DS-23 anti-PA reagent candidate produced after a streptavidin pre-sorting . Panel (4a) shows 68.8% binding to PA, and panel (4b) shows minimal cross-reactivity to streptavidin (1.6%). In all panels, the binding population is indicated in red.

Recent results routinely demonstrate peptide SREs with excellent specificity determined by evaluating relative binding to a cross-reactivity panel using flow cytometry and SPR analysis and we are currently extending reagent development to several toxin threats including Staphylococcal enterotoxin B (SEB). SEB is a 29-kDa superantigen and is one of the seven enterotoxins of *Staphyloccocus aureus* and is one of the most well understood and well studied staph enterotoxins.[5] Detection of the staphyloccocal enterotoxins in food is critical since food poisoning is often caused by enteric exposure of these enterotoxins, resulting in emesis and diarrhea[12]. In Figure 5(a), flow cytometry characterization of an isolated clone (peptide reagent candidate) shows a high binding affinity (K_d ~15 nm). The outstanding specificity that uniquely characterizes our SRE methodology is clearly evident upon comparison of relative binding to the SEB target and example protein from the cross-reactivity panel in Figure 5 (b) and (c), respectively.

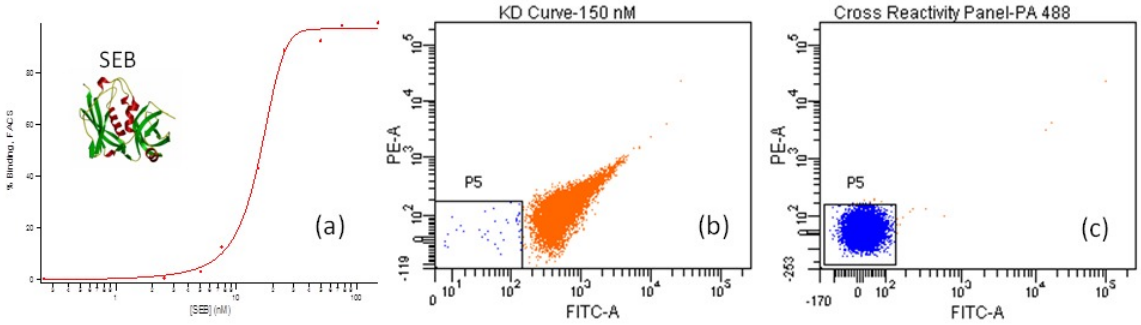

Figure 5: SEB toxin synthetic reagent performance characterization including a) Peptide binding study to 150 nM SEB showing ~15 nm Kd binding affinity, Inset: representation of SEB protein toxin (b) FACS analysis of peptide shows 99.7% binding of the reagent population to the target (orange), compared to the control (blue), and (c) FACS analysis shows minimal cross-reactivity (<1%) of the population to an equivalent concentration of protective antigen

Over the past year we have expanded our experimental success to include development of an integrative and iterative modeling capability to rapidly predict *smart* peptide binders for a target protein. Through the ARL developed XpairIt toolkit we have advanced the state-of-the-art in docking simulations incorporating a range of simulation methods to enable a more accurate assessment of protein-peptide interactions, ultimately providing predictive capability. The current computational suite combines a python-based application programming interface developed in-house, combined with state-of-the-art external software (VMD™, NAMD™,& PyRosetta™).

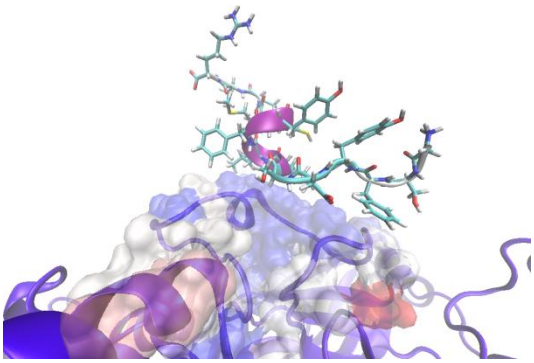

Figure 6: Atomistic level elucidation of binding mode enables a priori design of improved binders in future development rounds.

Figure 6 shows a peptide docked to protective antigen using the XPairIt simulator. The XPairIt Simulator (Version 2) was used to perform a blind test to predict and analyze the structure of a peptide synthetic reagent with the Anthrax PA bound complex. The simulation results highlight binding on domain 2 of the PA protein *a priori*, which were found to concur with experiment (data not shown). Simulation of the peptide-PA system was carried out with no knowledge of peptide secondary structure or binding location, a task notoriously difficult for other docking software. Figure 3 shows an example of the docked peptide with protective antigen after simulation. With the molecular dynamics and custom sampling methodology contained in XPairIt, the bound complex displayed an average interface energy increase of more than two-fold, and the frequency of hydrogen bonding increased by 26-fold when compared to standard Rosetta docking. The final XPairit simulator under development will include additional quantum mechanics, coarse graining and improved treatment of electrostatics. This is the first freeware, HPC-ready, extensible, biologically oriented multiscale modeling toolkit. The code is suitable for study of peptide-protein and protein-protein interactions and extensible to a variety of DoD applications (medical and non-medical).

4. CONCLUSION

To conclude, we highlight in this paper our approach to synthetic peptide reagent production based on bacterial display technology. We use a combination of semi-automated sorting and extensive characterization of relative binding affinity and specificity to isolate clones (i.e., peptide reagent candidates) with tailored binding performance. Biopanning, fundamentally is an affinity driven selection process. It is not surprising that peptide reagent candidates, and synthetic reagent candidates developed by similar methods such as phage display, are often characterized as high affinity binders, but are not very specific. In particular, "over-sorting" the library to yield only the highest binder affinity candidates is likely to yield these results. We have initiated the development of a multi-scale simulation toolkit capable of a priori docking simulations to enable *in-silico* screening of binder candidates, and ultimately predictions of new binder candidates with improved performance through *in silico* mutations. This coupled with discovery methodologies which favor specificity should ultimately allow development and evolution of reagents which satisfy stringent affinity and specificity performance requirements.

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